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A genetic system has been established for the isolation of candidate tumor suppressor genes in MCF7 breast cancer cells. This system includes the IPTG-inducible episomal vector, pEpiLac, which combines the advantages of inducible expression systems and extrachromosomal replication systems. pEpiLac vectors expressed exogenous genes more efficiently than non-episomal vectors. A cDNA library from normal human breast epithelial cells was constructed in the pEpiLac vector system. The LAP5 cell line, which supports inducible expression of the pEpiLac vector, was derived from human breast cancer MCF7 cells by the introduction of the LAP267 transactivator. Using the episomal vector system, we have conducted a selection for growth inhibitory cDNAs from breast epithelial cells in the LAP5-MCF7 breast cancer cell line. To date, about 12 candidate sequences have been isolated through two rounds of selections. These candidate tumor suppressors are now being characterized structurally and functionally.

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INTRODUCTION

Breast cancer is the most common malignancy observed among women in the Western hemisphere. It is estimated that one of every nine women in the United States will develop the disease. There is thus an urgent need to develop novel agents targeted for this disease. Many lines of evidence support the notion that breast cancer develop in part due to the loss of functional tumor suppressors, and that the introduction of a functional tumor suppressor gene can inhibit tumorigenicity and proliferation of breast cancer cells. Identification of such genes can lead to potential methods of treatment, including targeted gene therapy. Moreover, understanding the mechanism of action these tumor suppressors can lead to rationale drug design. We have developed a novel approach, called SETGAP (selectable expression of transient growth-arrest phenotype), to isolate cDNAs of genes from normal human breast cells that will suppress the growth of breast cancer cells. This approach allows the identification of growth suppressors of breast cancer cells using a direct functional assay, and no specific information of the genes to be isolated is necessary. This novel approach promises to open new doors towards understanding the molecular player that regulate breast cancer cell growth and towards potential gene therapy strategies.

The underlying hypothesis of this proposed project is that there exists previously unknown tumor suppressor genes that may be useful in the treatment of breast cancer and in the understanding of breast cancer etiology. A novel genetic selection procedure has been developed to identify and isolate gene transcripts present in normal human breast epithelium that are capable of inhibiting the growth of human breast cancer cells in culture. This novel approach may lead to the identification of previously unknown tumor suppressors, which may in turn open new doors for potential gene therapy and rational drug design.

RESULTS

Construction of pEpiLac vectors. The SETGAP procedure was designed to isolate potential growth inhibitory cDNA sequences (Pestov and Lau, 1994). One key component of this selection is the IPTG-inducible expression vector pX11, which contains 15 copies of *lac* repressor binding sites in front of the MMTV basal promoter. pX11 was further modified to generate pX12 through the mutation of Oct1 sites in the MMTV promoter; this modification decreased the basal level expression of the luciferase reporter gene (Pestov and Lau, unpublished data). To facilitate the selection procedure further, we have created episomal expression vectors based on the fact that the presence of EBV *ori*P region and EBNA-1 gene on the same DNA molecule allows for extra-chromosomal replication in primate cells (Yates et al., 1985). These episomal vectors have several advantages over other vectors, including high efficiency of stable transfection, high level expression of transfected genes, easy recovery of transfected sequences, and the low complexity of transfected sequences when a population of DNA sequences is introduced (Deiss et al., 1995). These advantages will make significant improvements upon the SETGAP procedure. Besides the high efficiency of stable transfection and high expression level of transfected genes, the convenient Hirt's extraction of transfected DNAs will eliminate the step to clone the error-prone PCR products from genomic DNAs. More importantly, it will be easier to pinpoint the growth inhibitory cDNAs from cells survived SETGAP selection because of the low complexity of transfected sequences. To pursue all these advantages, pEpiLac vectors were constructed as IPTG-inducible episomal expression vectors with different multiple cloning sites (MCS, Fig. 1).

Establishment of the MCF7/LAP5 cell line. LAP267 is a mammalian transcriptional activator which was derived by inserting VP16 transactivation domain after amino acid 267 of *lac*I repressor, and it also contains the SV40 nuclear localization signal (NLS) (Pestov and Lau, 1994; Baim et al., 1991). The transactivation activity of LAP267 was shown to be dependent on the presence of IPTG inducer (Pestov and Lau, 1994; Baim et al., 1991). In order to establish a cell line from the human breast cancer MCF7 to express LAP267 transactivator, MCF7 cells were cotransfected with pX6LAP267, which expresses LAP267 proteins, and pWLneo, which consistently expresses the neomycin-resistant gene. After G418 selection, 15 different clonal lines were analyzed. One clonal line, MCF7/LAP5 (called LAP5 hereafter), showed high level of IPTG-induced LAP267 transactivation activity, and this level is also dependent on the concentration of IPTG inducer. Thus, this cell line is appropriate for carrying out the SETGAP selection. Since we have now adapted the use of episomal expression vectors, the ease of plasmid recovery using Hirt's extraction method. Episomal DNAs could be conveniently isolated from a stable population of LAP5 cells transfected with pEpiLac1p27 using Hirt's extraction method. After amplification in bacterial cells, the integrity of these DNAs were confirmed by restriction enzyme digestion and PCR analysis (data not shown). Fig. 2 shows that expression of transfected sequences (detected by a luciferase reporter) is inducible by nearly 300 fold using the episomal vector transfected into LAP5 cells.

Improved SETGAP procedure. Since pEpiLac vectors efficiently express growth inhibitory genes in LAP5 cells and can be easily recovered using Hirt's extraction method, they are adapted into the SETGAP procedure in LAP5 cells. 1. A cDNA library is constructed by cloning cDNAs, in sense orientation, into the multiple cloning sites in pEpiLac vectors. 2. LAP5 cells are transfected with the library DNAs and selected against hygromycin. 3. Hygromycin selected

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cells are treated with IPTG to induce the expression of exogenous genes. 4. Proliferating cells are killed following BrdU/Hoechst dye/light selection, whereas, growth arrested cells will survive. 5. Growth arrested cells are rescued by the removal of IPTG inducer. 6. Episomal DNAs are extracted, amplified in bacteria and applied to the next round of selection. 7. After two rounds of selection, individual clones are applied to growth inhibition assays or DNA sequence analysis.

Selection of growth inhibitory sequences from a human breast epithelial cell cDNA library.
The first step was the construction of a cDNA library in pEpiLac1 using mRNAs from normal human mammary gland cells using standard techniques. LAP5 cells were transfected with the library DNAs and about 6,000 hygromycin-resistant clones were obtained. These cells were then selected through SETGAP. After one round of selection, extra-chromosomal DNAs were isolated from cells survived IPTG stimulation, amplified in *E. coli* DH10B cells, and applied to the second round of selection. The growth inhibitory effect of library DNAs was obvious after IPTG stimulation. No dramatic effect was observed for either parental LAP5 cells or cells transfected with the luciferase gene. Therefore, this subpopulation of cDNA library might contain some growth inhibitory sequences. After another round of selection, episomal DNAs were recovered from selected cells, amplified in bacteria and applied to another round of selection. Some cells were shown to be growth-arrested after IPTG stimulation. The sequences of these inhibitory clones are currently being determined. To date, 12 candidate clones have been isolated and are being characterized structurally and functionally. Fig. 3 shows that expression of the cDNA library sequences leads to growth inhibition, thus the rescue of cells from killing of proliferating cells. Expression p27^{KIP1}, a well-characterized growth inhibitor, was used as a positive control.

Progress with respect to the Statement of Work

The technical objectives originally proposed that cover the first year of work are as follows"

Task 1: Months 1-4:   Preparation of subtracted breast epithelial cell cDNA library
Task 2: Months 5-7:   Cloning of cDNA library into MCF-7 cells expressing LAP267; titrate conditions for genetic selection
Task 3: Months 8-11:   SETGAP selection of growth-inhibitory sequences in MCF-7 cells;first round selection
Task 4: Months 11-14: Second round selection of growth inhibitory cDNAs

We have accomplished much more than originally anticipated. In addition to having completed the second round selection of growth inhibitory cDNAs, we have also developed a new vector system that will facilitate the SETGAP selection. Thus, instead of the plasmid-based vector system, we have developed the episomal vector described above, which will facilitate the recovery of selected sequences considerably. We are now well on our way toward identifying growth inhibitory cDNAs and characterizing the tumor suppressive functions.

EXPERIMENTAL METHODS AND PROCEDURES

Plasmid constructs pLac vectors were derived from pX12 (Pestov and Lau, unpublished data) through two modifications. The multiple cloning site in pX12 was reconstructed with a pair of oligonucleotides containing three rare-cutting restriction enzyme sites, Fse I, Sfi I and Not I (Fig.1). Two other rare-cutting restriction enzyme sites (Sgf I and Srf I) were inserted into the Afl III site in the plasmid backbone. Proper sequences were confirmed through DNA sequencing. pEpiLac episomal vectors were constructed from pLac vectors and the episomal vector pREP4 (Invitrogene Corporation, Carlsbad, CA). pLac vectors were double digested with Afl III and Sac I; the cohesive ends were filled-in using Klenow fragments. pREP4 was digested with Sal I and the cohesive ends were also filled-in. The large fragment from pREP4 was ligated to the small fragment from pLac. The proper orientation was confirmed through physical mapping. pX6LAP267 is the auto-regulatory LAP267 expressing vector (Pestov et al., 1998). pWLneo consistently expresses the neomycin-resistant gene (Stratagene, La Jolla, CA). pHyg is the construct which consistently expresses the hygromycin-resistant gene (Sugden et al., 1985).

Cell culture MCF7 cells and MCF7/LAP5 cells were maintained, at 37°C and 5% CO_2, in modified Eagle's medium containing non-essential amino acids (Gibco BRL, Gaitherburg, MD), plus 1 mM of MEM sodium pyruvate (Gibco BRL), 1 mM of glutamine (Gibco BRL), 10% of fetal bovine serum (FBS, Intergen Company, Purchase, NY) and 10 μg/ml of bovine insulin (Gibco BRL). Cells were refreshed every 2-3 days. Where indicated, hygromycin (Boehringer Mannheim Corporation, Indianapolis, IN) or G418 (Gibco BRL) were added to mediums to the final concentrations of 75 μg/ml or 600 μg/ml respectively.

MCF7/LAP5 cells, called LAP5 cells in short, were derived from MCF7 cells by the cotransfection of pX6LAP267 (5 μg per 100 mm plate) and pWLneo (0.5 μg) using the calcium-phosphate precipitation technique. Two days after transfection, cells were replated into five 100 mm plates and selected against G418. The expression of LAP267 in individual clonal lines, including LAP5 cell line, was demonstrated by the IPTG-dependent expression of the luciferase reporter gene in transient transfection assays.

For serum stimulation, MCF7 cells were plated in 100 mm tissue culture dishes (1 X 10^6 cells each) in whole mediums and cultured for two days. Then, the cells were washed twice with PBS and cultured in phenol red free MEM (Gibco BRL) plus 1 mM of MEM sodium pyruvate (Gibco BRL), 1 mM of glutamine (Gibco BRL) and 0.1% FBS (Intergen Company); no insulin was added. Three days later, cells were changed back into whole mediums containing 10% FBS and incubated for indicated times. For UV irradiation, MCF7 cells, with mediums and covers of culture plates removed, were exposed to UV light in the Stratalinker (Stratagene) at 100 mJ/cm^2 and cultured in fresh mediums for indicated times.

Transfection For electroporation, 2 X 10^7 cells (in 0.8 ml of PBS, pH7.35) and 20 μg of DNA were incubated at RT for 10 min, and electrically pulsed, in a 0.4 cm cuvette at 960 μFD and 330 volts. After 5 min at RT, cells were cultured in fresh medium. For calcium phosphate precipitation, 7.5 μg of DNA and 5 X 10^5 cells were used for each 60 mm plate. The cells were exposed to DNA-calcium phosphate precipitates for 4-8 h and glycerol-shocked (15% glycerol in 20 mM HEPES-K^+, pH7.2) at RT for 1 min. Glycerol was removed by washing cells twice with PBS and cells were cultured in fresh medium and IPTG was added for 30-36 h where indicated.

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cDNA library construction Human mammary gland polyA$^+$ RNA (Clontech Laboratories, Palo Alto, CA) were utilized to synthesize cDNAs using the SuperScript system (Gibco BRL) with one modification to the manufacturer's guide. Double-stranded cDNAs were ligated to Fse I adaptors. The resulted cDNAs were cloned between Fse I and Not I sites in the multiple cloning site of pEpiLac1. Approximately 4 X 10^5 bacterial colonies were obtained after electroporation of DH10B competent cells with the ligation mixture. Colonies were pooled together, amplified in 2X YT medium at 30°C for 4 h. Plasmid DNAs were extracted using standard techniques. The library contains cDNA inserts averaging about 1 kb.

DNA extraction Episomal DNAs were isolated from LAP5 cells using Hirt's extraction method (Anant and Subramanian, 1992) with some modifications. Briefly, 1-2 X 10^6 cells were harvested in TEN buffer (40 mM Tris.HCl, pH7.5, 150 mM NaCl, and 1 mM EDTA), washed once with cold PBS, and then resuspended in 0.36 ml of ice cold TE buffer (10 mM Tris-HCl, pH 7.2, and 10 mM EDTA). Into the cell suspension, add 10% SDS to the final concentration of 1% and gently invert the tube several times. Incubate the sample at RT for 10-20 min, add 1/5 volume (80 µl) of 5M NaCl and mix the solution gently. Incubate at 4°C for more than 8 h or overnight. Centrifuge the samples at 14,000 rpm for at least 30 min at 4°C. Collect the supernatants, extract once each with phenol, phenol:chloroform, and chloroform:IAA(24:1). Precipitate the episomal DNAs with 0.3 M of sodium acetate and 2-3 vol of absolute alcohol, and dissolve the DNA pellet in water.

SETGAP selection The previously described SETGAP procedure (Pestov and Lau, 1994) was improved by the adaption of the IPTG-inducible episomal expression system into LAP5 cells. Briefly, 1 X 10^5 cells per 100 mm plate were cultured for 40-48 h in mediums containing none or 1 mM of IPTG (Sigma, Chemical Co., St. Louis, MO). Then BrdU (Sigma) was added to the final concentration of 15 µM in fresh medium. 40-48 h later, Hoechst 33342 (Calbiochem-Novabiochem Corporation, San Diego, CA) was added to 2.0 µg/ml. After 3 h incubation, tissue culture dishes were placed on a sheet of clear glass 5 cm directly above a 15 W fluorescent bulb (daylight type, Sylvania Electric Products, Fall River, MA), covered with aluminum foil, and irradiated from beneath for 30 min. Wash cells twice with PBS and culture in fresh medium for 3 days followed by 3 week incubation in hygromycin-containing mediums. Colonies were stained with 1% crystal violet (Sigma) in 20% ethanol.

RNA extraction and Northern blot analysis Total RNAs were isolated using the TriZol reagent (Gibco BRL) according to the manufacturer's guidance, and dissolved in 100% formamide (Fluka, Milwaukee, WI) to protect RNAs from degradation. RNAs were separated on 1% formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham Life Science, Little Chalfont, Buckinghamshire, England). To generate hybridization probes, pLac3 constructs containing indicated cDNA sequences were linerized with Sgf I digestion. Linerized DNAs were used to transcribe anti-sense RNA probes with the SP6 polymerase Strip-EZ kit (Ambion, Inc., Austin, TX) and ^{32}P-UTP (NEN, New England Nuclear, Boston, MA). Nylon membranes were incubated with the probe-labeling reaction mixture in the Zip-Hyb solution (Ambion, Inc.) at 65°C for 2 h, washed 2 X 15 min in 0.2 X SSC/0.2% SDS at RT, and 3 X 15 min in 0.1 X SSC/0.1% SDS at 80-85°C. After being rinsed in 2 X SSC buffer, the membranes were exposed to PhosphoImager screen (Molecular Dynamics, Sunnyvale, CA). After analyzing the data, membranes were striped with reagents in the Strip-EZ kit (Ambion) and re-probed, if necessary.

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PCR amplification PCR amplifications were performed with Pfu (Stratagene, La Jolla, CA) or Taq (Fisher, Pittsburgh, PA) DNA polymerases using M5 and P3 primers (Pestov and Lau, 1994). The reactions started with incubation at 94°C for 2 min. The cycle number was 25. For each cycle, DNA templates were denatured at 94°C for 1 min, annealed to primers at 55°C (for Taq) or 50°C(for Pfu) for 1 min, and amplified at 72°C for 3 min. Following the 25 cycles of amplification was the incubation at 72°C for 10 min.

CONCLUSIONS

A genetic system was established for isolating candidate tumor suppressor genes based on their growth inhibition phenotype. The IPTG-inducible episomal vectors, pEpiLac, were constructed. These vectors combined the advantages of inducible expression systems and extrachromosomal replication systems. The LAP5 cell line was derived from human breast cancer MCF7 cells by the introduction of the LAP267 transactivator. pEpiLac vectors expressed exogenous genes more efficiently than non-episomal vectors. Taking advantages of pEpiLac vectors and LAP5 cells, the SETGAP protocol is improved to make it easier to recover and identify exogenous growth arresting sequences.

The human breast cancer MCF7 cell line is one of the best-studied model systems in cancer research. Tightly controlled inducible gene expression will provide a mechanism for the study of tumor suppressor genes or cytotoxic genes. Based on the IPTG-dependent release of the *lac* repressor from the *lac* operator, Lee and colleagues tried to generate an IPTG-inducible expression system in MCF7 cells (Lee et al., 1997). But, the IPTG-induction of exogenous gene expression was not high; only a few fold of induction was reached (Lee et al., 1997). In this study, using the IPTG-dependent transactivator LAP267, we established the LAP5 clonal line from MCF7 cells. Exogenous genes can be overexpressed several hundred fold higher after IPTG stimulation.

SETGAP was design as and shown to be a practical method to isolate potential growth arresting DNA sequences (Pestov and Lau, 1994; Pestov et al., 1998). In this system, overexpression of growth arresting sequences inhibit mammalian cell growth; and cell proliferation can be rescued after the expression is turned down. In this study, the SETGAP procedure was improved by the adaption of the IPTG-inducible system to the pEpiLac episomal expression vectors. pEpiLac vectors express cDNA sequences more efficiently when a cDNA library is introduced into mammalian cells. After SETGAP selection, it is easy to recover the intact exogenous plasmids. After being amplified in bacteria, these episomal DNAs can be directly applied to the next round of selection or applied to the sequence characterization.

SETGAP procedure is used to isolate genes based on their growth inhibitory powers rather little about its normal physiologic role. The tumor suppressing activity of these genes need to be further analyzed based on tumor cell tumorigenic phenotype, as indicated by loss of anchorage independence and loss of the ability to generate tumors in nude mice. Tumor suppressor genes are frequently mutated and associated with loss of heterozygosity in inherited or sporadic cancers, studies of mutation status will also help determine tumor suppressing activities of isolated sequences, which were isolated from SETGAP.

A publication has already resulted from this study. This paper, entitled "Isolation of growth suppressors from a cDNA expression library" by Pestov, Grzeskiewicz, and Lau is in press in the journal *Oncogene*. A copy has been included in the Appendix.

Figure 1. IPTG-inducible episomal expression vectors, pEpiLac1 and pEpiLac3, which have different multiple cloning sites (MCS), as shown in panel B. *Ori*P, origion of plasmid replication of Epstein-Barr virus; EBNA1, Epstein-Barr virus nuclear antigen 1; Hyg, hygromycin resistant gene; lac, lactose operater sequences; MMTV-P, mouse mammary tumor virus basal promoter; SV40 polyA, SV40 virus polyadenylation signal; M5 and P3, PCR primers used to amplify inserts in the multiple cloning site; T7 and SP6, promoters which transcribe DNA sequences in sense orientation and anti-sense orientation, respectively.

A

Amp/ColE1

EBNA 1

tk-P

Hyg

tk polyA

pEpiLac
11.5kb

15Xlac

Ori P

MMTV-P

MCS

SV40 polyA
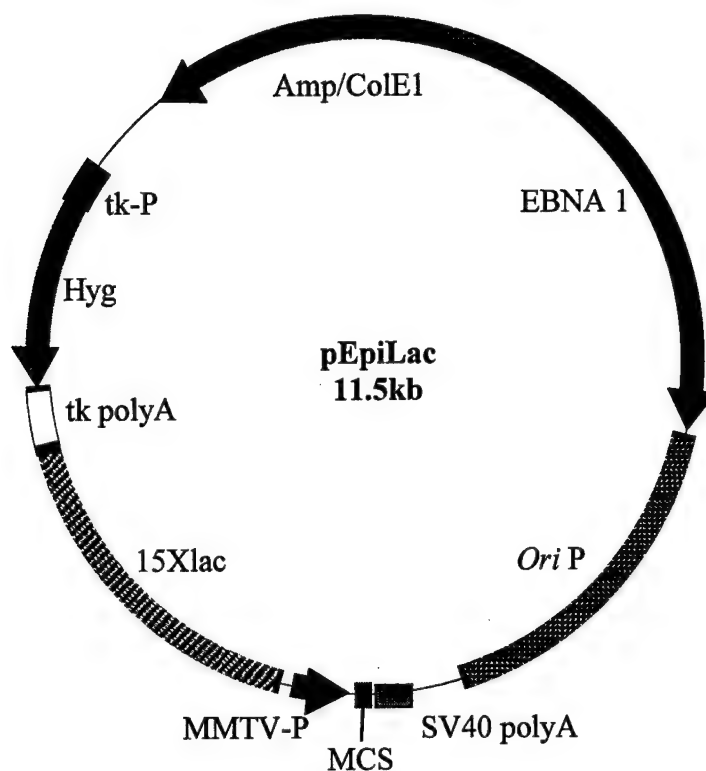
B

pEpiLac1:

M5⟶   Nhe I   Fse I   Sfi I   Not I   EcoR I

TCACCATAGGGACCAAGCTAGCGGCCGGCCTGCAGGGCCGCGGCCGCGGAATTCC

GGATAGGTAATTAGGGCCCCCTCGATCTTATTAAAGCA**GAACTTGTTTATTGCAGC**

                                            ◄——— P3

pEpiLac3:

M5⟶   T7⟶   Fse I   Nhe I

TCACCATAGGGACCAAGCTAGAtaatacgactcactatagggcgaGGCCGGCCGCTAGC

BamH I   Not I   ◄——— SP6   EcoR I

GGATCCGCGGCCGCgtattctatagtgtcacctaaatGGAATTCCGGATAGGTAATTAGGGCCC

CCTCGATCTTATTAAAGCA**GAACTTGTTTATTGCAGC**

                           ◄——— P3

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Figure 2. IPTG-induced expression of luciferase reporter gene in LAP5 cells. LAP5 cells were cotransfected with either pEpiLac1Luc (3 μg), pX12Luc (2 μg) or pLac1Luc (2 μg) together with pPGKβGal (1 μg) using calcium phosphate precipitation method. After being exposed to DNA precipitates for 8 h, cells were either left untreated or were treated with indicated amounts of IPTG for 32 h. Luciferase activities from these cells were measured and normalized with β-galactosidase activities. Representatives of three independent experiments were shown. The expression of the IPTG-dependent transactivator, LAP267, in LAP5 cells is demonstrated by the IPTG-induced expression of the luciferase reporter gene (A and B). The expression level of the luciferase gene is dependent on IPTG concentration (B). Episomal vectors, like pEpiLac1, could express luciferase gene more efficiently than non-episomal vectors, such as pX12 or pLac1 (A and B).
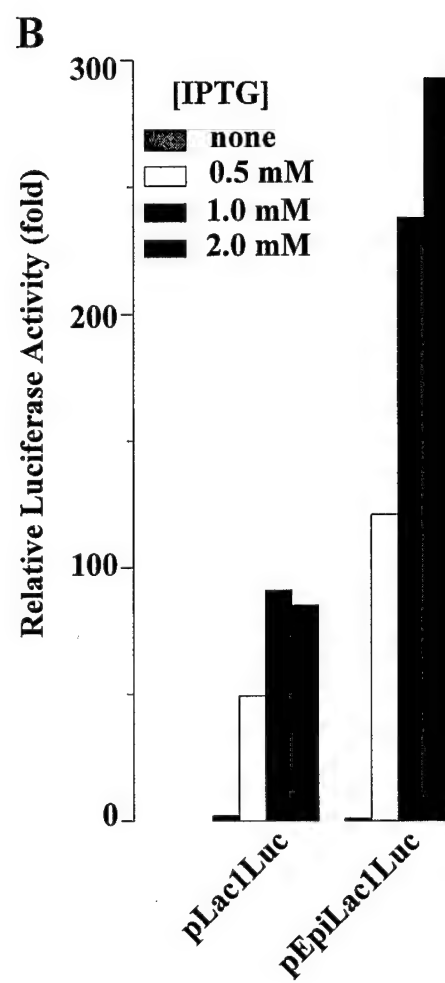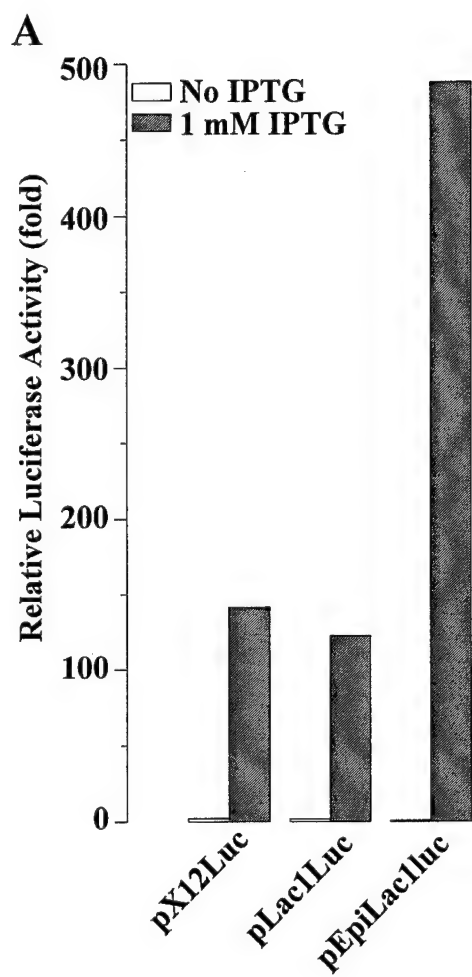
A

Relative Luciferase Activity (fold)

No IPTG
1 mM IPTG

pX12Luc pLac1Luc pEpiLac1luc

B

Relative Luciferase Activity (fold)

[IPTG]
none
0.5 mM
1.0 mM
2.0 mM

pLac1Luc pEpiLac1Luc

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Figure 3. Expression of potential growth inhibitory sequences in quiescent mouse fibroblast cells. A cDNA library was constructed in pX12 vector and applied to the previously described SETGAP procedure (Pestov and Lau, 1994) in LAP5 cells. Exogenous DNAs were PCR amplified and re-cloned into the pEpiLac1 vector in sense orientation. 20 μg of this mini-library DNAs or 20 μg of pEpiLac1p27 DNAs were applied to the improved SETGAP procedure (Fig. 11). This mini-library might contain some growth inhibitory sequences, since more cells survived SETGAP after IPTG treatment. Lib, cells transfected with library DNAs; p27[KIP1], cells transfected with mouse p27[KIP1] CDK inhibitor gene; LAP5, non-transfected LAP5 cells.

	LAP5	p27^{kip1}	Lib

-IPTG

+IPTG
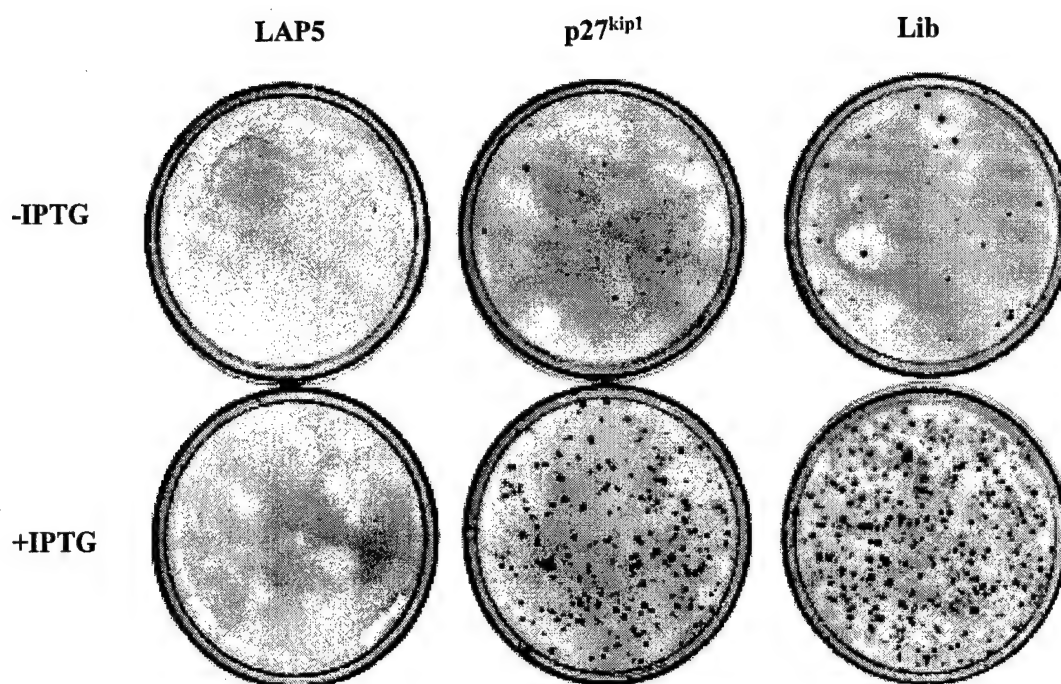
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Isolation of growth suppressors from a cDNA expression library

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We describe an experimental procedure for the isolation of growth inhibitory sequences from a complex cDNA library. This approach first takes advantage of the SETGAP technique (selectable expression of transient growth arrest phenotype) to enrich for growth inhibitory sequences, followed by a screening procedure to identify individual cDNAs that inhibit cell proliferation. Here we provide a detailed description of the experimental protocol and report the characterization of two cDNA sequences isolated in our initial screen of a mouse cDNA library. One of these cDNAs encodes the mouse ubiquitin-conjugation enzyme UbcM2. The other encodes a truncated form of a novel WD40 repeat protein, named Bop1, which is conserved from yeast to human. Together, these results demonstrate a new approach for the isolation of growth suppressors from cDNA libraries, and identify a previously unknown gene likely to be involved in growth control.

Keywords: inducible vector; Rb; 3T3 cells

Introduction

Cell proliferation can be regulated both positively and negatively. Considerable progress has been made in understanding the mechanisms that lead to activation of the proliferative program. Recent studies have also demonstrated the existence of multiple negative control mechanisms, which can intervene at several checkpoints to prevent cell cycle progression if previous cycle events have not been properly completed (Hartwell and Weinert, 1989; Elledge, 1996; Paulovich *et al.*, 1997). The importance of these mechanisms is underscored by the fact that the loss of function of the proteins involved, such as p53, can lead to genetic damage and neoplasia (Donehower *et al.*, 1992; Hartwell and Kastan, 1994; Cross *et al.*, 1995; Harper and Elledge, 1996). During development, most cells switch from active proliferation to nonreplicative, differentiated state. Although growth-arrested cells constitute the majority of cells in the organism, the molecular mechanisms that establish and maintain their replicative arrest are only poorly understood.

Positive regulators of cell growth may confer a growth advantage on cells expressing them, or may indeed overpower the cellular growth control machinery and lead to uncontrolled proliferation. Consequently, a plethora of oncogenes have been identified by means of retroviral transduction and gene transfer.

In contrast, the identification of genes that can suppress proliferation in a dominant manner has proven more difficult. Cells expressing a gene that cause growth arrest may be eliminated from a growing culture, and these cells do not have a phenotype for which a selection can be easily applied. The use of genetic methods for studying growth-inhibitory pathways has therefore been limited.

We have recently developed a method that allows the isolation of growth-inhibitory DNA sequences by genetic selection in mammalian cells (Pestov and Lau, 1994). This method, called SETGAP (selectable expression of transient growth-arrest phenotype), utilizes a library that potentially contains growth-inhibitory sequences cloned in an inducible expression vector. After transfection into mammalian cells, expression of library DNA is transiently induced to block growth of those cells that have incorporated growth-inhibitory sequences. These cells are then isolated from the transfected population by selective killing of cells that are capable of DNA replication.

In the previous study, we tested the feasibility of SETGAP in the selection of short inhibitory fragments of a small group of known growth-related genes (Pestov and Lau, 1994). In an effort to devise a method to identify previously unknown growth-inhibitory genes, we have developed a two-step procedure based on SETGAP. Using this approach, we have isolated two cDNA clones that can reversibly arrest cell growth. One of these clones encodes a known gene, the mouse ubiquitin-conjugating enzyme UbcM2. The other encodes an amino-terminal truncated sequence of a novel gene with unknown function, *bop1*. Expression of both of these cDNAs in NIH3T3 fibroblasts results in a reversible inhibition of G1 progression. These results suggest that our experimental approach can be a useful strategy for identification of novel growth-inhibitory genes in cDNA libraries.

Results

Genetic selection for the enrichment of growth inhibitory sequences

We have developed a two step approach to isolate growth-inhibitory cDNA clones from a complex cDNA library. First, we applied the SETGAP selection procedure (Pestov and Lau, 1994) to enrich for sequences that are capable of arresting cell growth when expressed. Second, we subjected the enriched cDNA sequences to a screening procedure to identify individual growth-inhibitory clones.

As a source of mRNA for our cDNA library, we used primary mouse embryo fibroblasts cultured

according to the 3T3 regimen to the point of crisis, whereupon these cells lost their proliferative potential and took on morphological characteristics reminiscent of senescent cells (Todaro et al., 1965; Todaro and Green, 1963). mRNA from these fibroblasts was used to prepare a cDNA library using the vector pX11, which allows IPTG-inducible expression in NIH3T3-derived LAP3 cells (Pestov and Lau, 1994). The cDNA library was transfected into LAP3 cells; approximately 10^4 stably transfected clones were divided into several pools and subjected to selection using a modified SETGAP procedure that was adapted for a complex full-length cDNA library. The essential steps in this selection are as follows (Figure 1): (1) Cells that incorporate BrdU while transfected sequences are expressed under IPTG induction are selectively killed; (2) Cells that are growth-inhibited due to expression of transfected sequences survive the selective killing and are rescued by the removal of IPTG. Another cycle of IPTG induction and BrdU/light treatment can be applied to the rescued cells to reduce background; (3) Transfected cDNA sequences are recovered by PCR and recloned into pX11. Since cells rescued after one round of selection may contain multiple sequences because of cotransfection, sequences recovered from them can be pooled into secondary libraries and subjected to another round of selection to further reduce complexity. After the second round, the rescued sequences can be analysed individually.
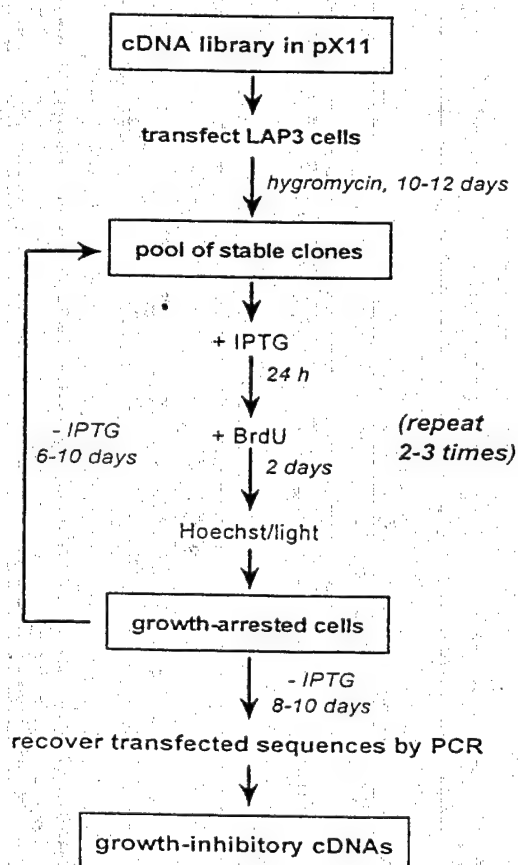
LAP3 cells transfected with the cDNA library were treated with IPTG to induce expression of the transfected sequences, which resulted in increased survival of cells after BrdU/light treatment (Figure 2a). A similar increase was observed when LAP3 cells were transfected with a growth-inhibitory variant of JunB (11-11) (Pestov and Lau, 1994), whereas cells transfected with negative controls (pX11 empty vector or pX8B6-luc) did not show a similar effect. Cells that survived one round of selection were expanded and transfected sequences were recovered by PCR, generating mixtures of products that ranged from 300 bp to >3 kb in length. The rescued sequences were subjected to another round of selection. To this end, PCR products >700–800 bp in length recovered from surviving cells from each dish were isolated from an agarose gel and recloned into pX11 to create a secondary library. Twenty secondary libraries thus obtained were transfected into LAP3 cells, generating a few thousand stable clones in each transfection. Four out of the 20 secondary libraries were positive in SETGAP selection, yielding surviving cells. The transfected sequences recovered by PCR from these cells were significantly less complex than cDNAs isolated after one round of selection. However, attempts to reduce this complexity further by additional rounds of selection were unsuccessful, most likely due to unavoidable cotransfection of multiple sequences into a single cell in the calcium phosphate-mediated transfection procedure.

Screening for individual growth-inhibitory cDNA clones

Sequences recovered from each of the four cell populations that survived the second round of selection were cloned into pX11 and 30–50 individual plasmid clones were isolated at random. Plasmid DNA of each clone was digested with HinfI to produce a distinct pattern to exclude identical clones from further analysis. In order to identify individual growth inhibitory sequences, we devised a screening procedure, SETGAP-2. Clones were cotransfected with a β-galactosidase marker plasmid into LAP3 cells, which were subjected to one cycle of IPTG induction and BrdU/light treatment and then stained with X-gal (technical details are provided in Materials and methods). The growth of blue colonies surviving the BrdU/light procedure indicated the growth-inhibitory potential of the transfected sequence. Using this screening method, we isolated two growth-inhibitory clones derived from two separate cell populations. The two isolated growth-inhibitory clones contained inserts of 1.2 kb (clone 23F4) and 1.75 kb (clone B5-35).

Expression of 23F4 or B5-35 inhibits cell proliferation

Expression of 23F4 or B5-35 in LAP3 cells led to a dramatic increase in the number of cells that survived the SETGAP procedure (Figure 2b). To confirm that this effect was due to growth inhibition, we pooled cells from approximately 10^3 stable clones derived by transfection with 23F4, B5-35 or control constructs pX11 and pX11-p27, and counted the number of cells after three days of culture in the presence or absence of IPTG (Figure 3). Pools of transfected cells rather than clonal cell lines were examined in this experiment to
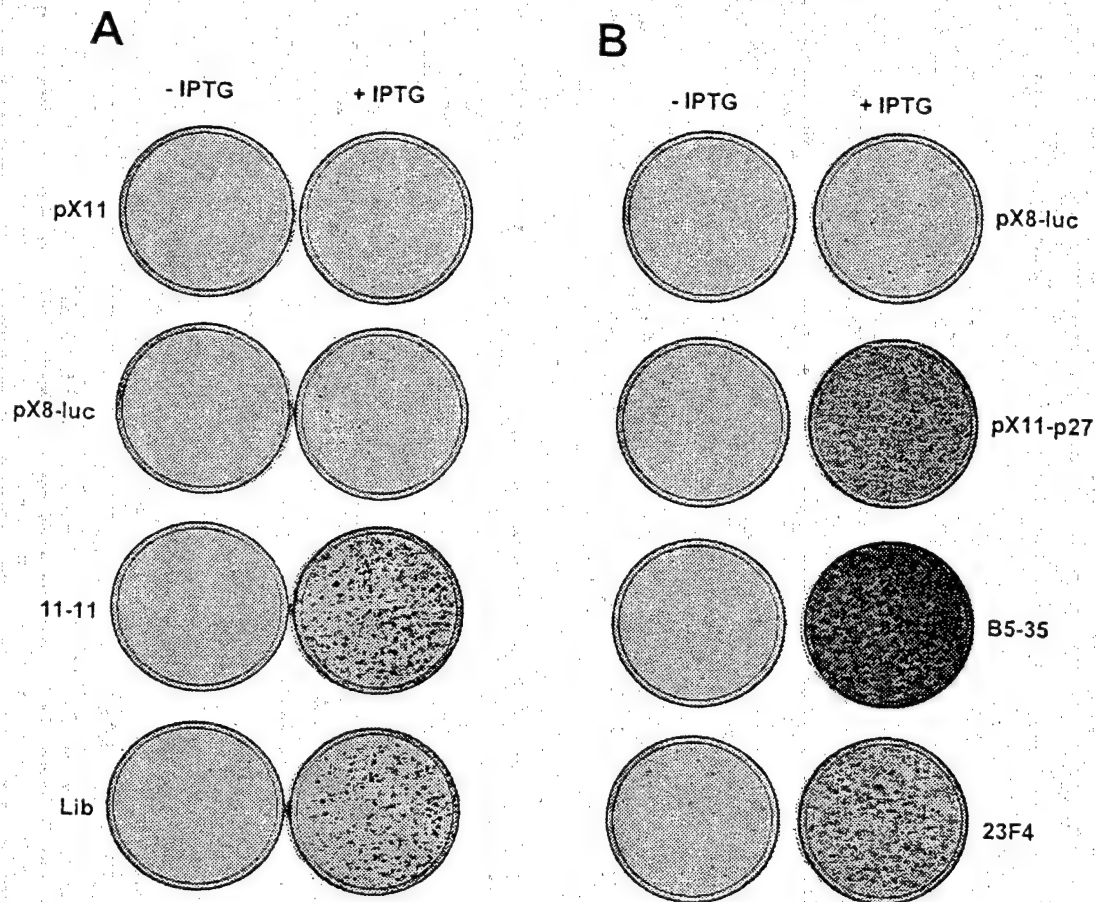

Figure 1 Schematic representation of the SETGAP procedure for selection of growth-inhibitory cDNA sequences

A

- IPTG   + IPTG

pX11

pX8-luc

11-11

Lib

B

- IPTG   + IPTG

pX8-luc

pX11-p27

B5-35

23F4

Figure 2 SETGAP selection for growth inhibitory cDNAs. (a). Cells were transfected with either the cDNA library (Lib), the growth-inhibitory construct 11-11 (a truncated variant of JunB) (Pestov and Lau, 1994), or the negative control plasmids pX11 (empty vector) and pX8-luc (vector driving expression of the luciferase gene). Stable clones obtained from each transfection were pooled, and 10^5 cells from each pool were subjected to two cycles of BrdU/light treatment to kill cells that synthesize DNA. Cells were tested for survival of BrdU/light treatment either in the presence or absence of transfected sequences. After the second BrdU/light treatment, surviving cells were allowed to grow for 7 days in the absence of IPTG and then stained with crystal violet. (b) Cell survival after BrdU/light treatment upon induction of transfected sequences (+IPTG). Cells were transfected with either the negative control pX8-luc, the positive control pX11-p27 (a p27Kip1 expressing plasmid), and clones B5-35 and 23F4. Experiments were carried out as in (a), except that cells were subjected to only one cycle of BrdU/light treatment before surviving cells were rescued

eliminate any clonal effects on growth rates. While the presence of IPTG did not significantly alter the growth of cells transfected with pX11, induction of B5-35 resulted in a significant decrease in the rate of cell proliferation, comparable to the effect of expression of the transfected cdk inhibitor p27Kip1 (Figure 3). Induction of expression of 23F4 in this assay resulted in a modest inhibition of the growth rate, which was consistent with a relatively smaller effect observed in SETGAP (see Figure 2b). When we first subjected the pool of cells transfected with 23F4 to one round of SETGAP selection and used the surviving cells for the cell proliferation assay, these pre-selected cells displayed a clear reduction in their growth rate upon IPTG treatment (Figure 3). The observed increase in the growth-inhibitory effect after SETGAP selection likely reflects the enrichment in the proportion of cells that express the 23F4-encoded sequence at a sufficiently high level to cause growth inhibition. Parallel selection of cells transfected with the inducible p27Kip1 construct also increased the observed growth-inhibitory effect of its expression (Figure 3). Taken together,

these results show that expression of B5-35 and 23F4, identified in a SETGAP assay, can indeed inhibit proliferation in LAP3 fibroblasts.

Expression of B5-35 or 23F4 leads to G1 arrest

To investigate the nature of the growth inhibition conferred by expression of cDNA sequences in B5-35 and 23F4, we cotransfected these plasmids with pHyg into LAP3 cells and generated stable clonal lines in which expression of the transfected sequences was inducible by IPTG (Figure 4). We tested the effect of expression of these sequences on the ability of cells synchronized by serum starvation to initiate DNA synthesis. Several independently obtained stable lines were serum-starved and then restimulated with 10% serum in the presence of BrdU. The number of cells entering S phase was assessed by immunohistochemical staining of BrdU-positive nuclei. Induction of B5-35 and 23F4 in several tested lines inhibited the ability of cells to enter S phase, whereas no effect was observed in the control cell line transfected with the empty
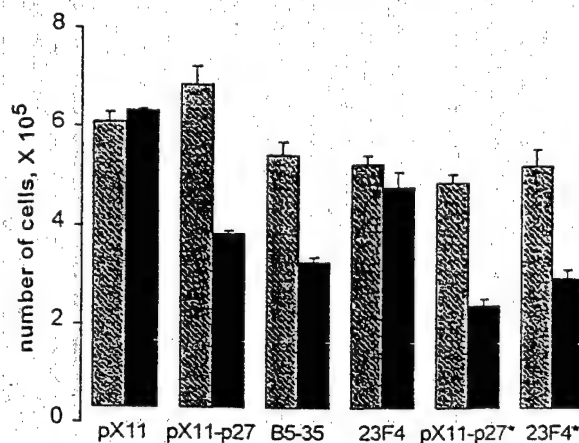
Figure 3 Expression of B5-35 or 23F4 inhibits cell proliferation. Stable clones obtained by transfection of indicated plasmids into LAP3 cells were pooled and seeded into 35-mm dishes (3×10^4 cells per dish). Cells were counted after 3 days of growth, before cells reached saturation density, in medium with 1 mM IPTG (solid bars) or without IPTG (shaded bars). Error bars show variations among duplicate dishes. Two sets of bars (pX11-p27 and 23F4) labeled with an asterisk (*) show data obtained with transfected cells pre-selected with one round of SETGAP
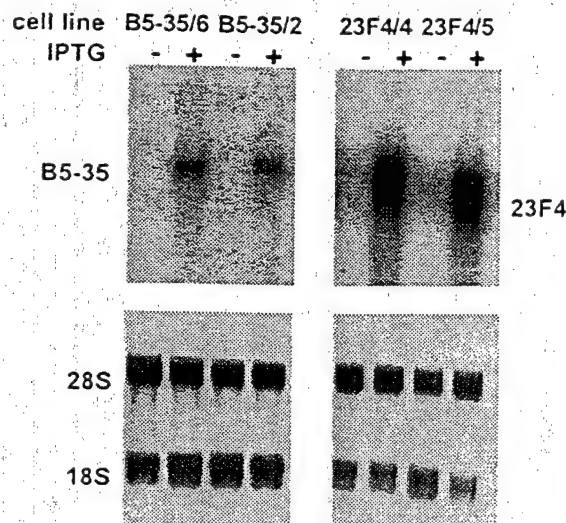

Figure 4 Inducible expression of B5-35 and 23F4 mRNA in clonal cell lines. Individual clones of LAP3 cells stably transfected with B5-35 (lines B5-35/6 and B5-35/2) or 23F4 (lines 23F4/4 and 23F4/5) were either untreated (−) or treated (+) with 1 mM IPTG for 9 h. Total RNA (2.5 μg for left panel and 1.5 μg for right panel) was analysed by Northern blotting with the corresponding cDNA probes. Lower panel: equal loading in each lane was shown by methylene blue staining of the same blot to reveal ribosomal RNAs

To test whether the inhibition of S phase entry might be due to G1 arrest, we analysed the status of the retinoblastoma tumor suppressor protein (Rb), which is hyperphosphorylated prior to G1 exit, in inducible cell lines B5-35/6 and 23F4/4. These cells were synchronized by serum starvation and then stimulated with 10% serum. Induction of B5-35 with IPTG strongly inhibited Rb phosphorylation (Figure 6). Although Rb phosphorylation was not inhibited in cells expressing 23F4, it is considerably delayed (compare hypophosphorylated Rb at 12 h in 23F4-expressing and -non-expressing cells). These data indicate that the antiproliferative effects of both B5-35 and 23F4 are, at least in part, due to restriction of progression through G1 prior to Rb phosphorylation.

Clone 23F4 encodes a ubiquitin-conjugation enzyme

Sequence analysis revealed that clone 23F4 encodes the entire reading frame of the mouse ubiquitin-conjugating enzyme UbcM2. Compared to the previously published UbcM2 sequence (Genebank accession no. X92664), 23F4 (Genebank accession no. AF003346) differs by a single A→G substitution within the coding region (nucleotide 94 in 23F4) and contains a longer 3′-untranslated region. The nucleotide substitution was not due to a mutation that occurred during SETGAP selection, since it was also present in a sequence independently obtained from primary mouse fibroblasts by reverse transcription-PCR using UbcM2-specific primers.

To determine whether growth inhibition by de-regulated expression of UbcM2 is dependent on its enzymatic activity, we created a single amino acid substitution mutant construct in which the cysteine-145 residue at the active site of this enzyme (Matuschewski et al., 1996) was replaced with a serine residue. This mutation abolished inhibition of cell growth as judged by the SETGAP assay, suggesting that it is the enzymatic function of UbcM2 that causes the growth inhibition (Figure 7), rather than any non-productive interactions that may result from overexpression of the protein.

Clone B5-35 identifies a novel WD40 repeat protein, Bop1

We isolated a full-length cDNA sequence corresponding to B5-35 from a mouse library as described in Materials and methods. The isolated cDNA, designated Bop1 (block of proliferation), was 2476 bp long, in agreement with the size of a single transcript detected in mouse cells. The B5-35 clone (1737 bp) initiates at nt 747 of the bop1 sequence, having deleted the 5′ end (Figure 8). The B5-35 cDNA contains a single long open-reading frame that starts with two closely located ATG codons, the second of which (nt. 799; Figure 8) is in a good Kozak context. We constructed an expression vector encoding a deletion mutant, Bop1Δ, whose translation is initiated with this codon. Expression of Bop1Δ led to growth inhibitory effects indistinguishable from those of B5-35 (Figure 7). When tested in SETGAP, the full-length Bop1 construct inhibited proliferation of LAP3 cells, indicating that increased levels of Bop1 can induce cell cycle arrest (Figure 7). The effect of Bop1

vector (Figure 5). Expression of B5-35 (line B5-35/6) and 23F4 (line 23F4/4) blocked DNA synthesis in 80% of cells in this experiment; expression of 23F4 in another clonal line, 23F4/5, resulted in approximately 50% fewer cells undergoing DNA synthesis. In all stable cell lines examined, induction of either 23F4 or B5-35 was not apparently toxic and did not induce any noticeable increase in cell death even after a prolonged IPTG treatment.
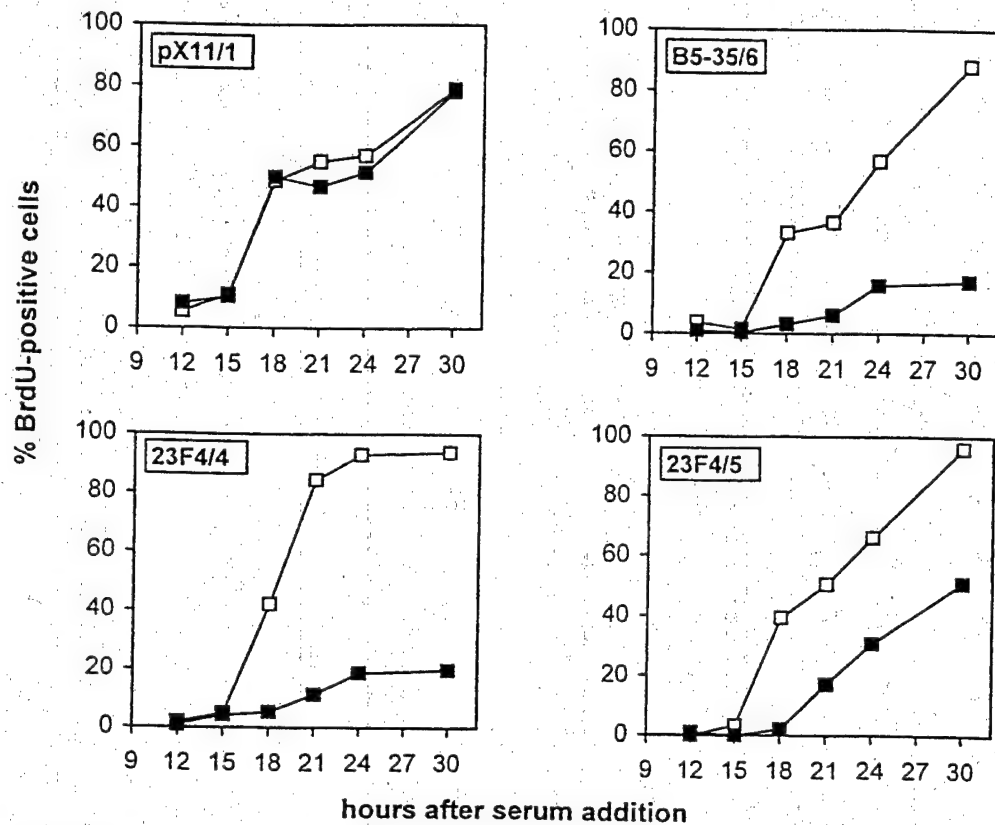
Figure 5 Expression of B5-35 or 23F4 inhibits S phase entry in serum-stimulated cells. Clonal lines transfected with either the empty vector (pX11/1), or B5-35 (B5-35/6)) or 23F4 (23F4/4 and 23F4/5) were plated in chambers on glass slides and starved in 0.1% serum for 72 h. Cells were re-stimulated with 10% serum in medium containing 10 μM BrdU. Cells were either treated (■) or untreated (□) with IPTG (1.5 mM) 6 h prior to serum stimulation, and again at the time of serum addition. At the indicated times following stimulation (12–30 h), cells were fixed and stained with anti-BrdU antibodies. The percentages of BrdU-positive nuclei were determined by observing at least 200 cells at each time point. An independent experiment monitoring [^{3}H]-thymidine incorporation showed similar results

expression, however, was not as striking as that of B5-35. The simplest interpretation of this fact is that the deletion of the amino-terminal part of Bop1 creates a gain-of-function mutant. Another possibility is that the amino-terminal deletion in B5-35 might create a dominant-negative mutant that is more potent in perturbing Bop1 function than overexpression of the full-length protein. Further studies will be needed to determine the physiologic role of Bop1 and to understand how interference with its function results in a strong, albeit reversible, block to cell cycle progression.

Database analyses revealed two sequences that are homologous to Bop1: one in human (KIAA0124), and one in the yeast S. cerevisiae (YM9796.02c) (Figure 8). The human KIAA0124 sequence was obtained by sequencing randomly chosen human cDNA clones and contains a partial coding sequence (Nagase et al., 1995). The yeast sequence YM9796.02c was deduced from an open reading frame found in the genome of this organism. All three amino acid sequences are highly homologous except in their amino-termini. The deduced mouse Bop1 shares approximately 45% amino acid sequence identity with the yeast YM9796.02c, and >90% amino acid identity with the human sequence, excluding the amino-terminus (Figure 9). The amino-terminal domains of all three of these proteins are divergent in their primary structure, although they are
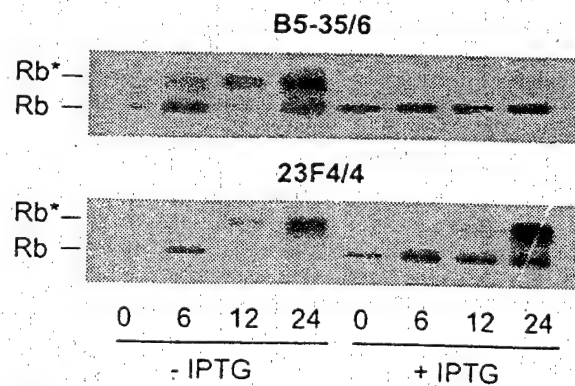
Figure 6 Phosphorylation of Rb is inhibited or delayed in cells expressing B5-35 or 23F4. The cells lines B5-35/6 and 23F4/4 were made quiescent by incubation in 0.1% serum for 40 h. Cells were then stimulated with 10% serum for the indicated times (hours) either in the presence of absence of 1 mM IPTG (added 16 h prior to serum stimulation where indicated). Cell lysate was prepared and Rb was detected by Western blotting (see Materials and methods). Rb*, hyperphosphorylated form of Rb

very similar in composition. The most prominent feature of these domains is that they are rich in acidic and serine residues and contain PEST sequences, which are often found in short-lived regulatory proteins (Rechsteiner and Rogers, 1996).

| pX11 | 23F4 | 23F4mut | Bop1 | Bop1Δp14 |

Figure 7 Effects of mutations in UbcM2 and Bop1. Pools of cells stably transfected with either the empty vector (pX11), or expression constructs of UbcM2 (23F4), a cysteine-145 to serine mutation of UbcM2 (23F4mut), the full length *bop1* (Bop1), and 5' deletion of bop-1 (Bop1Δ) were analysed in the SETGAP assay. Expression of transfected sequences was induced with IPTG, and cells were subjected to BrdU/light treatment. Growth of surviving cells was restored by IPTG removal and the resulting colonies were stained 9 days thereafter
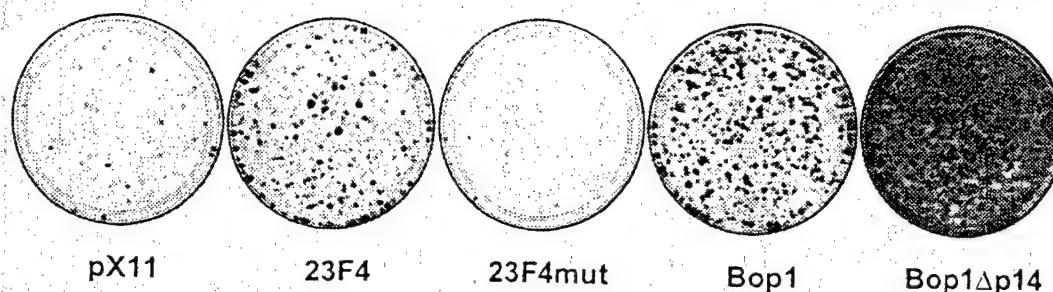
The amino acid sequence of Bop1 contains several motifs, known as WD40 (or β-transducin) repeats (Figure 8), which are found in a large number of regulatory proteins (Neer *et al.*, 1994; Neer and Smith, 1996) and may be involved in protein-protein interactions (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996). The WD40 motif encompasses approximately 40 amino acid residues with several conserved features, including a character-istic Trp-Asp (WD) dipeptide. All WD40 repeat proteins contain from 4 to 10 repeats that display different degrees of divergence from the consensus WD40 motif structure (Neer *et al.*, 1994). Four WD40 repeats were identified in Bop1 by a pattern search against the Blocks database (Pietrokovski *et al.*, 1996). Repeats 1 and 4 (Figure 8) are close to the consensus structure, while repeats 2 and 3 are more divergent. Interestingly, repeat 3 contains a Cys residue instead of the canonical Trp (or another aromatic) residue in the marker WD dipeptide, although it gains a high score in a computer search as a true WD40 repeat due to proper positioning of other characteristic amino-acid residues.

Discussion

The SETGAP procedure was developed as a genetic approach to isolate growth-inhibitory sequences based on a functional assay in mammalian cells (Pestov and Lau, 1994). This procedure was first applied in the selection of short genetic suppressor elements targeted at a small group of known growth-related genes. In this report, we describe an adaptation of the SETGAP procedure for the cloning of growth-inhibitory cDNA sequences from a complex library. This adaption takes advantage of the genetic selection conferred by SETGAP to enrich for growth inhibitory sequences, followed by a screening procedure to identify individual clones.

We started by constructing a conditional expression cDNA library using mRNA from mouse fibroblast passaged to crisis; the cDNA library was transfected into LAP3 cells and an estimated 10⁴ of the transfected clones were first subjected to selection to enrich for growth-inhibitory cDNA sequences. Each of the transfected clones typically contained 10-100 cDNA sequences, as judged by PCR analysis (data not shown). After two consequtive rounds of SETGAP selection, the PCR pattern of cDNA recovered from cells was simple enough to permit analysis of individual clones.

In this study, we carried out our selection and screening with a relatively small number (10⁴) of transfected clones. Given that typically 10-100 sequences are incorporated into each clone, the actual number of sequences that have been tested in this screening is much larger than the number of clones screened. However, we speculate that the two growth inhibitory cDNAs we isolated in this screening represents only a small fraction of such sequences in the library for several reasons. First, the transfected sequences must be expressed at an appropriate level under induction to cause transient growth arrest. Second, we encountered a relatively low success rate in recovery of growth-inhibitory cDNA sequences from growth-arrested cells during selection (about 20% in this study). This may be due to a loss of integrity of some cDNA sequences upon chromosomal integration, which could prevent their amplification by PCR. Third, it is possible that in some cells growth arrest is caused by combinatorial or additive action of multiple sequences, and this effect may be lost when the sequences are tested individually. We also noted that some cDNA sequences chosen at random for testing were refractory to amplification by Pfu, although they could be efficiently amplified by other enzymes. To improve the recovery of cDNA sequences from transfected cells, we now use PCR protocols that utilize a combination of polymerases.

Most of the problems that limit the efficacy of the present experimental procedure derive from the nature of transfection in mammalian cells. Perhaps a different method of introduction of cDNA into cells (for instance, using virus-based or episomal vectors) might improve the success rate in the selection step. Some of the aforementioned problems can be addressed by screening a larger number of clones, and in this regard, reduction of the complexity of the starting library should greatly enhance the efficacy of the procedure. Recently, highly efficient methods for normalization of cDNA libraries have been developed (de Fatima Bonaldo *et al.*, 1996). The significantly lower overall complexity of such libraries should make it possible to analyse a large fraction of the cDNAs.

One of our original concerns in application of expression cloning for the isolation of growth-inhibitory cDNA was that this approach might primarily yield cDNA clones that encode proteins that have no clear role in growth control but nevertheless cause growth inhibition when their expression is out of balance relative to other cellular components. In fact, many 'structural' genes (such as actin, β-tubulin, actin-binding protein, nonhistone protein B) were isolated in yeast screens for overexpression-induced growth suppressors (Liu

et al., 1992). However, we have not identified any structural gene sequences in our search for growth inhibitory sequences to date. Screening of small groups of clones (10–100 sequences each) from the original cDNA library, also did not yield such sequences, even though abundant transcripts should be represented among these clones (data not shown). A possible reason for this is that previous screening procedures (such as screens in yeast) identified growth-inhibitory sequences based on induction of a lethal phenotype. In contrast, the SETGAP

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CTCCCGCTGC CGGAAGCGGC AGTAGATCGG TAGCCGGCGC GGCGCAGCAT GGCGGGGGCG TGTGGTAAGC CCCACATGTC ACCGGCATCG CTGCCGGGGA    100
                                                          M  A  G  A  C  G  K  P  H  M  S  P  A  S  L  P  G
                           |α8 start
AACGACGTTT GGAACCTGAT CAGGAGCTGC AGATACAAGA GCCTCCTCTC CTCAGCGATC CTGACTCCAG TCTCTCTGAC AGCGAGGAGA GTGTGTTTTC    200
K  R  R  L  E  P  D  Q  E  L  Q  I  Q  F  P  P  L  L  S  D  P  D  S  S  L  S  D  S  E  E  S  V  F  S
AGGCCTCGAA GATTTGGGCA GTGACTCGAG TGAGGAAGAC ACTGAAGGAG TGGCCGGATC CAGCGGTGAC GAAGACAACC ATAGGGCAGA GGAGACCTCT    300
 G  L  E  D  L  G  S  D  S  S  E  E  D  T  E  G  V  A  G  S  S  G  D  E  D  N  H  R  A  E  E  T  S
GAGGAGCTGG CACAGGCTGC CCCTCTTTGC TCAAGGACAG AGGAAGCAGG TGCTCTGGCC CAAGATGAAT ATGAAGAGGA CAGCTCTGAT GAGGAGGACA    400
 E  E  L  A  Q  A  A  P  L  C  S  R  T  E  E  A  G  A  L  A  Q  D  E  Y  E  E  D  S  S  D  E  E  D
TTCGGAACAC TGTGGGCAAT GTGCCCCTGG CATGGTACGA TGAATTCCCA CATGTGGGTT ATGACCTGGA TGGCAAACGT ATCTACAAGC CCCTGCGGAC    500
I  R  N  T  V  G  N  V  P  L  A  W  Y  D  E  F  P  H  V  G  Y  D  L  D  G  K  R  I  Y  K  P  L  R  T
ACGAGATGAG CTCGACCAGT TTCTGGACAA AATGGATGAC CCAGATTTCT GGCGCACTGT GCAAGACAAG ATGACAGGGC GTGATCTGCG GCTAACTGAT    600
R  D  E  L  D  Q  F  L  D  K  M  D  D  P  D  F  W  R  T  V  Q  D  K  M  T  G  R  D  L  R  L  T  D
GAGCAGGTTG CCCTGGTACA CCGGCTTCAG AGAGGCCAGT TCGGAGATTC AGGCTTCAAT CCCTATGAGC CAGCTGTGGA CTTCTTCAGC GGTGACATCA    700
E  Q  V  A  L  V  H  R  L  Q  R  G  Q  F  G  D  S  G  F  N  P  Y  E  P  A  V  D  F  F  S  G  D  I
                                              |B5-35 start                                    (T)
TGATCCACCC TGTGACCAAC CGCCCAGCTG ACAAGCGTAG TTTCATCCCA TCCCTAGTTG AAAAGGAGAA GGTGTCTCGA ATGGTACATG CCATCAAGAT    800
M  I  H  P  V  T  N  R  P  A  D  K  R  S  F  I  P  S  L  V  E  K  E  K  V  S  R  M  V  H  A  I  K  M
GGGGTGGATC AAGCCTCGAC GGCCCCACGA CCCCACCCCT AGCTTCTATG ACCTGTGGGC CCAGGAGGAT CCGAATGCTG TGTTGGGACG CCACAAGATG    900
 G  W  I  K  P  R  R  P  H  D  P  T  P  S  F  Y  D  L  W  A  Q  E  D  P  N  A  V  L  G  R  H  K  M
CATGTGCCTG CCCCCAAGCT GGCCTTGCCT GGCCATGCCG AGTCTTACAA CCCCACCTCC GAGTACCTGC CCACTGAGGA GGAGCGGCTCG GCGTGGATGC    1000
 H  V  P  A  P  K  L  A  L  P  G  H  A  E  S  Y  N  P  P  P  E  Y  L  P  T  E  E  E  R  S  A  W  M
AGCAGGAGCC TGTGGAGAGG AAGCTTAACT TCTTGCCACA GAAATTCCCC AGCTTGAGGA CAGTGCCTGC TTACAGCCGC TTCATCCAGG AGCGTTTCGA    1100
Q  Q  E  P  V  E  R  K  L  N  F  L  P  Q  K  F  P  S  L  R  T  V  P  A  Y  S  R  F  I  Q  E  R  F  E
GCGCTGCCTC GATTTGTATC TGTGCCCACG GCAACGCAAG ATGAGGGTGA ATGTGGACCC CGAAGACCTC ATCCCCAAAC TCCCTCGGCC AAGAGACCTT    1200
 R  C  L  D  L  Y  L  C  P  R  Q  R  K  M  R  V  N  V  D  P  E  D  L  I  P  K  L  P  R  P  R  D  L
CAGCCTTTCC CTGTGTGCCA GGCCCTCGTC TACAGGGGCC ACAGCGACCT TGTCCGTTGC CTCAGTGTCT CGCCAGGGGG CCAGTGGCTA GCTTCAGGTT    1300
Q  P  F  P  V  C  Q  A  L  V  Y  R  G  H  S  D  L  V  R  C  L  S  V  S  P  G  G  Q  W  L  A  S  G
CAGACGATGG CACGTTGAAG CTCTGGGAGG TGGCCACTGC CCGCTGTATG AAGACTGTAC ATGTTGGAGG TGTGGTGCGG AGCATCGCCT GGAACCCCAA    1400
S  D  D  G  T  L  K  L  W  E  V  A  T  A  R  C  M  K  T  V  H  V  G  G  V  V  R  S  I  A  W  N  P  N
TCCTACCATA TGCCTGGTAG CTGCTGCCAT GGATGATGCC GTGTTGCTGC TGAACCCAGC CCTGGGGGAC CGGCTGCTGG TGGGCAGCAC AGACCAGCTG    1500
 P  T  I  C  L  V  A  A  A  M  D  D  A  V  L  L  N  P  A  L  G  D  R  L  L  V  G  S  T  D  Q  L
CTGGAAGCCT TCACTCCACC TGAGGAGCCA GCTCTGCAGC CTGCCCGGTG GCTAGAGGTC TCGGAGGAAG AGCACCAGAG GGGTCTGCGC CTACGCATCT    1600
L  E  A  F  T  P  P  E  E  P  A  L  Q  P  A  R  W  L  E  V  S  E  E  E  H  Q  R  G  L  R  L  R  I
GCCACAGCAA ACCAGTGACA CAGGTGACCT GGCATGGGCG AGGGGACTAT TTGGCGGTGG TGCTGTCTAG TCAAGAGCAC ACGCAAGTGC TGCTTCACCA    1700
C  H  S  K  P  V  T  Q  V  T  W  H  G  R  G  D  Y  L  A  V  V  L  S  S  Q  E  H  T  Q  V  L  L  H  Q
GGTGAGCAGG AGGCGCAGCC AGAGCCCCGTT CCGCCGCAGC CACGGGCAGG TGCAGTGTGT GGCCTTCCAT CCCTCCCGGC CCTTCCTGCT CGTGGCCTCC    1800
 V  S  R  R  R  S  Q  S  P  F  R  R  S  H  G  Q  V  Q  C  V  A  F  H  P  S  R  P  F  L  L  V  A  S
CAGCGCAGCA TCCGCATTTA CCACCTGCTG CGCCAGGAGC TAACCAAGAA GCTGATGCCC AACTGCAAGT GGGTGTCCAG CATGGCTGTA CATCCAGCAG    1900
Q  R  S  I  R  I  Y  H  L  L  R  Q  E  L  T  K  K  L  M  P  N  C  K  W  V  S  S  M  A  V  H  P  A
GTGACAACAT CATCTGTGGC AGCTATGACA GCAAACTGGT GTGGTTTGAC CTGGATCTTT CCACCAAGCC ATACAAAGTG CTGAGGCACC ACAAGAAGGC    2000
G  D  N  I  I  C  G  S  Y  D  S  K  L  V  W  F  D  L  D  L  S  T  K  P  Y  K  V  L  R  H  H  K  K  A
CTTGCGGGCT GTGGCCTTCC ACCCCCGATA CCCACTCTTT GCATCCGGCT CAGACGACGG CAGTGTTATC GTGTGCCATG GCATGGTGTA CAATGACCTG    2100
 L  R  A  V  A  F  H  P  R  Y  P  L  F  A  S  G  S  D  D  G  S  V  I  V  C  H  G  M  V  Y  N  D  L
CTGCAGAACC CATTGCTGGT GCCTGTCAAG GTGCTGAAAG GACACACCCT GACCCGAGAT CTGGGTGTTC TGGATGTGGC CTTCCACCCC ACACAGCCGT    2200
L  Q  N  P  L  L  V  P  V  K  V  L  K  G  H  T  L  T  R  D  L  G  V  L  D  V  A  F  H  P  T  Q  P
GGGTCTTCTC CTCCGGGGCA GATGGCACCA TTCGACTCTT CAGCTAAGCC AACACACTAC TCTGGGTGGC CTGGAAGAGG GTGGGTGTGC CTCCACACAG    2300
W  V  F  S  S  G  A  D  G  T  I  R  L  F  S  *
AATCCTCAAT AGAATCCCAT TCTTCCTGTC TTCCTGATTC AGCCTCCAGC AACTGCCCAG CCTTGACCTT ACACAGCACC CAGAACCTGG CTTAAGCAG    2400
AAGGGAAGGT GGTATTGATA CAATGTCACC CACTGAGGTG TAACTGGCTT TATTGGGCAA TAAATTGGGG CACCACAAAA AAAAAAAAAA AAAAA
```

Figure 8 Nucleotide and deduced amino acid-sequences of the *bop1* cDNA. The full-length *bop1* cDNA encodes a protein of 732 amino acids containing four WD40 repeats (underlined). The PEST sequence in the amino-terminus is underscored by a dash line. The starting positions of clones B5-35 and z8 are indicated by arrows. The two ATG codons at the start of the B5-35 open reading frame are at nt 781 and 799. (T) denotes a single-base substitution found in B5-35 when compared to the sequence of clone a8 isolated from the cDNA library. Asterisks indicate in-frame stop codons

method makes use of the formation of colonies by surviving cells, which requires two events: (i) stringent blockade of DNA replication upon induction of transfected sequences, lasting for the duration of BrdU treatment (typically 48 h), and (ii) reversal of the growth arrest when expression of transfected sequences is downregulated. Hence, factors that can slow down metabolism but incapable of causing a prolonged cell cycle block or sequences that are toxic for a cell would not be identified in SETGAP. We anticipate that this feature of the SETGAP method should facilitate the identification of regulatory genes. In support of this idea, tests with several known genes capable of arresting the cell cycle in a reversible manner showed that they can be readily identified by the

SETGAP procedure (for example, see results with p27Kip1, Figure 2b).

The two growth-inhibitory cDNAs isolated in this study were 1.2 and 1.75 kb in length. Since the majority of the mRNAs in the cell are between 1–2 kb, we expect that many growth-inhibitory cDNA sequences can be successfully recovered using the present experimental protocol. One of the isolated clones, 23F4, encoded a full coding sequence of UbcM2, while clone B5-35 contained a partial deletion of Bop1 cDNA, which fortuitously created a very strong growth-inhibitory sequence. The isolation of partial cDNA fragments is not surprising, as many clones in any cDNA library are products of incomplete cDNA synthesis. Although this may initially appear a disadvantage, the isolation of

```
Bop1       : MAGACGNPHMSPASLPGKRFLEPDCELQIKEPPLLSDPDSSLSDSEESVFSGHEDLGSDSSEEDTEGVAGSSGDFDNHPA  :  80
KIAA0124   : -----------------------------------------------------CSGSDSSEDDDFGDEGEGGAIDDEGHSGI  :  30
YM9796.02c : ---MMAKNNKTTEAKMSKKPAASEFSDVEEDEDKILS-----------VDGTIDAEASESDEDDDEYESAVEEKESSSD   :  65

Bop1       : EETSEELAQAAPLCSRTEEDGALAQDE------------------------------------------------YE   : 109
KIAA0124   : KKTEEQVQASTPQPRTEMASPRIGDE-------------------------------------------------YA   :  59
YM9796.02c : KEAQDDSDDDSDAELNKLLAEEEGDGESDYDSSEFSDDTTSLTDRLSGVKLQTIVDPNIYSKYADGSDRIIKPEINPVYD   : 145

Bop1       : EDSSDEEDIRNTVGNVPLANYDEFPHVGYDLEGKRIYKPLRTRDELDQFLDKMDDPDFWRTVQDKMTGRDLRLTDEQVAI   : 189
KIAA0124   : EDSSDEEDIRNTVGNVPIEJYDCFPHVGYDLEGRRIYKPLRTRDELDQFLDKMDDPDYWRTVQFENTGPDLRLTDFQVAI   : 139
YM9796.02c : SDDSDAET-QNTTGNIPISAYDIMPHIGYDINGKRIMREAHG-SALDQLLESIELPEGATGIIDFNSGSSLNLTKEEIER   : 223

Bop1       : VHRLQRGQFGLSGFNPYEPAVDFFSGE--IMIHPVTNRPADKRSFIPSLVEKEKVSRMVHAIKMGWIKF-------RRFH   : 260
KIAA0124   : VRRLCSGQFGDVGFNPYEPAVDFFSGI--VMIHPVTNRPADKRSFIPSLVEKEKVSRMVHAIKMGWICG-------RRFR   : 210
YM9796.02c : ISKIQPNEQTEDSINPYEPLIDWFTRHEEVN--PLTAVEEPKFRFVPSKMEAKRVMKIVPAIFECPIIEPKKLKEMKEKE   : 301

Bop1       : DPTPSFYDLWAQEDPNAVLGRHKMHVPAPKLALPGHAESYNPPPEYIPTEEEPSAWNQQEFVERKLNFLPQKFPSLFTVE   : 340
KIAA0124   : DFTPSFYDLWAQEDPNAVLGRHKNHVPAPKLALPGHAESYNPPPEYLLSEEEFLAWEQOEFGERKLSFLFRKFPSLFAVF   : 290
YM9796.02c : KIENYCYDLWG---DSTETNDHVMHLRAPKLPPGTNEESYNPFEEYLLSPEEKEAWENTEYSEFERNFIPCKYSALRKVE   : 378

Bop1       : AYSRFIQERFERCLDLYLCPRQRKMRVNVDPEDLIPKLPRPRDLQPFPVCQALVYRGHSDLVRCLSVSPGGQWLASGSDI   : 420
KIAA0124   : AYGRFIQERFERCLDLYLCPRQRKMRVNVDPEDLIPKLPRPRDLQPFFTCQALVYRGHSDLVRCLSVSPGGQWLVSGSDI   : 370
YM9796.02c : GYCESIRERFEFSLDLYIAPEVRPNKLNIDPNSLIPELFSPKDIRPFPIRCSTIYAGHKGKVFTLSICFSGLWLATGSDI   : 458

Bop1       : GTLKLWEVATARCMKTVHVGG-------VVRSIAWNPNFTICLVAAANDIAVLLLNF----------------ALGDRI   : 476
KIAA0124   : GSLRLWEVATARCVRTVPVGG-------VVKSVAWNFSEAVCLVAAAVEISVLLLNF----------------ALGDRI   : 426
YM9796.02c : GTVRVWEILTGPEVYRTTLIDDEENPDYHIECFEWNPIANNGILAVAVGENIHLIVEPIFGYDIENNGKTKIEDGFGYDT   : 538

Bop1       : LVGSTDQLLEAFTPF---SEFA--------LQPARWLEVSEEFHQRGLRLRICHSKPVTQVTWHGRGDYLAVVISSCFH   : 544
KIAA0124   : VAGSTDQLISAFVPP---SEFP--------LQPARWISASEEFQVGLRLRICHGKPVTQVTWHGRGDYLAVVIATGGH   : 494
YM9796.02c : FGTVKKSNLEVNENGDGDEDGENESAKNAVKKCVFCWNKPSGKGLEHCICITMSCKFTVKRLSWHPKGDYFVTVQQPDSGN  : 618

Bop1       : TQVLLHQVSRRRSQSPFRRSHGQVCCVAFHFSRPFLLVASQRSIRIYHLLRQELTKKLMPNCKWVSSMAVHPAGDNIICG   : 624
KIAA0124   : TQVLIHQLSRRRSQSPFFHSHGQVCRVAFHFARPFLLVASQRSVRLYHLLRQELTKKLMPNCKWVSSLAVHPAGDNVICG   : 574
YM9796.02c : TSVLIHQVSFHLTQSPFKKSRGIIMDAKFHFFKFQLFVCSQPNVRIYDLSCGILVKRLLFGARWLSKIDEHFRGDNLIAS   : 698

Bop1       : SYDSKLVWFDLDLSTKFYKVLRHHKKALRAVAFHFRYPLFASGSDDGSVIVCHGMVYNDLLQNPLLVPVKVLKGHTLTRI   : 704
KIAA0124   : SYDSKLVWFDLDLSTKPYRMLRHHKKALRAVAFHFRYPLFASGSDDGSVIVCHGMVYNDLLQNPLLVPVKVLKGSVLTRI   : 654
YM9796.02c : SFDKRVLWHDLDLASTFYKTLFYHEKAVPSVNFEKFLPLFSSAFDDGTIHVFHATVYDDMMRNPMIVPLFFLTGHMVINS   : 778

Bop1       : LGVLDVAFHPTQPWVFSSGADGTIRLFS------------------  : 732
KIAA0124   : LGVLDVIFHPTQPWVFSSGADGTVRLFT------------------  : 682
YM9796.02c : LGVLDAIWHEREAWLFSAGALNTARLWT------------------  : 807
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Figure 9 Comparison of amino acid sequences of the murine Bop1 and its human (KIAA0124) and yeast (YM9796.02c) homologs. Alignment was compiled using the Clustal W program (Thompson *et al.*, 1994) and modified by using GeneDoc (Nicholas *et al.*, 1997). Shading indicates identical positions and conservative substitutions

truncated cDNA sequences in a selection for growth inhibition can also be informative. For instance, identification of dominant negative variants of regulatory genes essential for cell replication could reveal practical ways to inactivate such genes. It is conceivable that some partial cDNAs isolated by this method could encode activated forms of growth-inhibitory proteins. In this regard, we note that many of the genes identified by their ability to induce cell transformation in traditional gene transfer experiments represent activated forms of their normal cellular variants.

The specific cellular processes affected by over-expression of the uniquitin-conjugating enzyme UbcM2 (clone 23F4) that can lead to inhibition of cell cycle progression are currently unknown. The ubiquitin degradation system is known to regulate a variety of cellular processes (Liu *et al.*, 1992; Hochstrasser, 1995), including the turnover of many proteins that control cell growth, including cyclins (Seufert *et al.*, 1995), c-Myc (Ciechanover *et al.*, 1991), p53 (Scheffner *et al.*, 1993; Maki *et al.*, 1996) and others (Bai *et al.*, 1996; Schneider *et al.*, 1996). UbcM2 belongs to a group of evolutionarily conserved ubiquitin-conjugating enzymes of the E2 class with different amino-terminal extensions (Matuschewski *et al.*, 1996). Overexpression of UbcM2 in yeast can partially suppress deficiency of UBC4 and UBC5 (Hochstrasser, 1995), which are involved in stress response and degradation of regulatory and abnormal proteins (Seufert and Jentsch, 1990 ; Chen *et al.*, 1993). These data suggest that enzymatic activity of UbcM2 could affect degradation of a regulatory protein(s) whose activity is critical for cell cycle progression.

The B5-35 sequence encodes a fragment of a novel WD40-containing protein, Bop1, truncated from the amino-terminus. Interestingly, expression of this truncated form caused a stronger cell cycle arrest than overexpression of the full-length cDNA. Additional studies will be needed to determine the mechanism of the growth-inhibitory activity associated with expression of Bop1 and whether the B5-35 clone acts by a dominant-positive or negative mechanism with regard to the full-length clone. Several lines of observation suggest that Bop1 may function as a regulatory protein. First, all other WD40 proteins studied to date have been implicated in regulatory activities (Neer *et al.*, 1994; Neer and Smith, 1996). Second, the presence of PEST sequences suggests that Bop1 may be short-lived, also pointing to possible regulatory functions (Rechsteiner and Rogers, 1996). Finally, the structural conservation of Bop1 from yeast to human suggests that function of this protein may also be conserved in diverse eukaryotic cells.

In summary, we have developed a method for the isolation of growth-inhibitory cDNAs from cDNA libraries. This experimental procedure could find application in identification of new cell cycle inhibitors and discovery of antiproliferative genes that may regulate the resting state in quiescent and senescent cells. This genetic strategy could potentially yield a class of growth-inhibitory molecules that would be difficult or impossible to isolate based on biochemical studies of known regulatory pathways.

Materials and methods

Cell culture

Cell culture. LAP3 cells (Pestov and Lau, 1994), an NIH3T3 cells-derived cell line which constitutively expresses the IPTG-regulated transactivator protein LAP267 (Baim *et al.*, 1991), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) and penicillin-streptomycin (GIBCO-BRL). Cells were transfected by the calcium phosphate coprecipitation method (Chen and Okayama, 1988) using 2.5 μg of library DNA or 0.5 μg of a specific construct, 0.5 μg of pHyg (Sugden *et al.*, 1985) and 2.5–4.5 μg of carrier NIH3T3 DNA per 60-mm dish. Stable clones were selected in 130–150 μg/ml^{-1} hygromycin (Boehringer Mannheim). Expression of transfected constructs was induced by the addition of 1–1.5 mM IPTG (dioxane-free, Sigma) to culture medium.

Plasmids

The inducible vector pX11 designed for low background expression, the inducible luciferase reporter pX8B6-luc and growth-inhibitory clone 11-11 (a fragment of *junB*) have been described previously (Pestov and Lau, 1994). The murine cyclin/cdk inhibitor p27(Kip1) was cloned into pX11 to generate pX11-p27; induction of this construct with IPTG inhibited growth of LAP3 cells (Shiyanov *et al.*, 1997).

Construction of the inducible cDNA library

Mouse embryo fibroblasts were derived from gestational day 17 embryos of Swiss mice by standard techniques (Loo *et al.*, 1989). The cells were passaged according to the 3T3 regimen (Todaro and Green, 1963) in DMEM containing 10% calf serum and penicillin-streptomycin. Cells were counted at each passage to monitor the decline in replicative potential. At the beginning of the crisis stage, their RNA was isolated by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Poly(A) RNA was purified using PolyATtract System (Promega).

cDNA synthesis was carried out using reagents from the SuperScript system (GIBCO-BRL) with several modifications to the manufacturer's protocol. Prior to cDNA synthesis, two oligonucleotides, N3 (<u>CTAGC</u>TGCGGACGAACATTACA) and BR3 (<u>CCGGA</u>ATTCGCATGAGAGTATA), were designed to incorporate partial *Nhe*I and *Bsp*EI sites (underlined) into the 5' and 3' ends of the cDNA, respectively. These primers were later used in PCR rescue of cDNA sequences from transfected cells. The first cDNA strand was synthesized from 5 μg of poly(A) RNA using 200 pmoles of GA-BR3-T16 primer ((GA)$_7$TCCGGAATTCGCATGAGAGTATA(T)$_{16}$). A nucleotide mixture containing 10 mM each of dATP, dGTP and dTTP (Pharmacia) and 6 mM of 5-methyl-dCTP (Boehringer Mannheim) was used instead of the 10 mM dNTP mixture supplied with the SuperScript kit. After the first strand synthesis, dCTP was added to 400 μM for the second strand synthesis. Ligation of double-stranded cDNA with N3 adapters and phosphorylation were performed according to the SuperScript system protocol. The cDNA was then digested with *Kpn*2I (an isoschizomer of *Bsp*E I that does not cut methylated DNA), extracted with phenol-chloroform and size fractionated on a Sephacryl S-500 column supplied with the kit. The resulting cDNA was ligated into pX11 digested with *Nhe*I and *Bsp*EI. Approximately 8 × 10^5 bacterial colonies were obtained by electroporation of DH10B cells (GIBCO-BRL) with the ligated cDNA. The colonies from 35 plates were scraped into 100 ml of SOB medium and incubated with shaking at 30°C for 1 h. The plasmid DNA was isolated using standard techniques.

The library was estimated to contain $> 5 \times 10^5$ clones with an average insert size of 1.2–1.5 kb.

BrdU/light selection of growth-arrested cells

10^5 stably transfected LAP3 cells were plated into a 10-cm dish in medium containing 1 mM IPTG to induce expression of sequences cloned into pX11. After 24 h, BrdU (Sigma) was added to a final concentration of 10 mM and cells were cultured for 2 days. Hoechst 33258 (Sigma) was then added to 10 μg/ml^{-1} for 1 h and cells were irradiated with visible light for 10 min as described previously (Pestov and Lau, 1994). Alternatively, a 10 min incubation with 1 μg/ml^{-1} Hoechst 33342 (Calbiochem), which was as effective as the longer treatment with Hoechst 33258, was used. The medium was removed after irradiation and cells were rinsed and refed with fresh medium. Medium was changed on days 2 and 4 after irradiation to remove dead cells.

Rescue of transfected DNA from cells after selection

Cells that survived BrdU/light selection were grown for 8–10 days until they formed small colonies. Genomic DNA was isolated using DNAzol reagent (Molecular Research Center, Inc.) followed by additional treatment with RNase A prior to PCR amplification with Pfu (Stratagene), a high fidelity polymerase (Lundberg et al., 1991; Bej and Mahbubani, 1994). DNA was first amplified using M5 and P3 primers, which flank the cloning site in the pX11 vector (Pestov and Lau, 1994), and then reamplified with N3 and BR3 primers. PCR was carried out in a Robocycler (Stratagene) using the following program: initial denaturation at 99°C for 10 s; cycling at 99°C for 20 s, 50°C (M5 and P3 primers) or 55°C (N3 and BR3 primers) for 40 s, 68°C for 6 min. These parameters allowed efficient amplification of sequences of up to 4 kb. After 16–18 cycles of PCR with M5 and P3 primers, 1–2 μl from this reaction was directly used in a second-step PCR with N3 and BR3. The number of cycles in this PCR was determined empirically for each template, since it dramatically affected the outcome of the reaction. Increasing the number of cycles often shifted the bias of amplification toward smaller fragments. Although supplying additional Pfu polymerase (1.25 U per 25 μl reaction) helped alleviate this problem, it was important to keep the number of cycles to a minimum, enough to only visualize DNA after electrophoresis of a 5 μl aliquot (1/5–1/10 of the total reaction volume) on an agarose gel.

PCR products were extracted with phenol-chloroform and purified using Microcon-100 concentrators (Amicon), followed by treatment with T4 polynucleotide kinase and then with exoIII (Kaluz et al., 1992) to expose NheI and BspEI ends. This DNA was separated on a low melting point agarose gel; fragments larger than 700–800 bp were purified from the agarose using GELase (Epicentre) and ligated into pX11 DNA that had been digested with NheI and BspEI and dephosphorylated. For isolation of secondary libraries, $> 10^4$ bacterial colonies obtained by electroporation of DH10B cells were scraped from plates, and processed as described above for the primary library. Individual clones were isolated using Qiawell-8 system (Qiagen).

Screening for growth-inhibitory clones ('SETGAP-2')

LAP3 cells grown in a 6-well cluster were cotransfected with a cDNA clone to be tested and pGK-β-gal. After transfection, cells were kept in the same well. After 5–6 days, cells were trypsinized and split into two dishes (1/5 of cells from one well to one 100 mm dish). The waiting period before starting the assay was necessary to relieve non-specific growth inhibition immediately after

transfection. One dish was kept without further treatment and used to control the transfection efficiency by staining the cells with X-gal. To the second dish, IPTG was added and BrdU/light treatment was performed as described above. Five or 6 days after this treatment, cells were stained with X-gal. Cells were washed once with PBS, fixed with cold 0.5% glutaraldehyde in PBS, rinsed twice with PBS and incubated for 2–3 h at 37°C in staining solution (1 mM MgCl$_2$, 3.3 mM of each K$_4$Fe(CN)$_6$ and K$_3$Fe(CN)$_6$, 0.1% X-gal and 0.02% NP-40 in PBS).

Other analytical methods

To analyse Rb phosphorylation, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8), 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma) for 30 min at 4°C followed by centrifugation for 10 min at 10 000 g. Lysates (~ 50 μg of total protein) were first concentrated by immunoprecipitation and then analysed by immunoblotting using the G3-245 antibody (Pharmingen). Detection of secondary horseradish peroxidase-coupled antibodies (Amersham) was performed with the LumiGLO reagents (KPL).

For immunohistochemical determination of BrdU incorporation, cells cultured in Falcon Culture Slides were fixed with cold 70% ethanol and allowed to air-dry. Slides were rehydrated in water for 30 min, treated with 2N HCl for 30 min, rinsed with 0.2 M Tris-HCl (pH 8), PBST (0.2% Tween-20 in PBS), blocked with 10% fetal calf serum in PBST for 10 min, washed twice with PBST, incubated for 1 h with peroxidase-conjugated anti-BrdU antibodies (Boehringer Mannheim), washed three times with PBST, stained using DAB-Plus kit (Zymed), counterstained with hematoxylin and covered with 70% glycerol. The percentage of BrdU-labeled cells was determined using a light microscope.

Cloning and sequence analysis

Nucleotide sequences were determined using Sequenase (USB) and EXCEL (Epicentre) reagent kits and protocols. To clone the full-length Bop1 cDNA, the cDNA library described above was screened with a B5-35 probe, and the longest isolated clone of 2345 bp (designated α8) was sequenced. Both B5-35 and α8 clones contained poly(A) tails at their 3' ends at almost identical positions (six extra nucleotides were present in B5–35). The sequence of B5-35 differed from the corresponding fragment of the library-derived clone α8 by a single base substitution (C→T at nt 791 resulting in a Ala→Val change; Figure 7). The 5' end of the cDNA was cloned using the 5' Rapid Amplification of cDNA ends (RACE) System (GIBCO BRL). The sequence of α8 was combined with 131 bases derived by sequencing several independent 5' RACE clones into a sequence of 2476 bp. This sequence is likely to contain the full coding sequence since it corresponds in size to the Bop1 transcript, contains an in-frame initiating ATG and in-frame termination codon upstream. The sequences of clone 23F4 and full-length Bop1 have been deposited into the Genebank (accession numbers AF003346 and U77415, respectively). An expression construct for Bop1Δ was created by using PCR primers to synthesize the bop1 cDNA that initiated with nt 799 of the bop1 sequence (Figure 7), resulting in a 5' truncation. The sequence of the truncated bop1 was confirmed by sequence determination and cloned in pX11. A cysteine-145→Serine mutation (23F4Mut) in UbcM2 was created by a T→A transversion at nt 436 of the 23F4 sequence.

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